Nitric oxide signaling in plants: cross-talk with Ca^{2+}, protein kinases and reactive oxygen species

Jéremy Astier[#1], Angélique Besson-Bard[#1], Izabela Wawer[1,3], Claire Parent[2], Sumaira Rasul[1], Sylvain Jeandroz[4], James Dat[2] and David Wendehenne[1]

[1]UMR INRA 1088/CNRS 5184/Université de Bourgogne, Plante-Microbe-Environnement, 17 rue Sully, BP 86510, 21000 Dijon, France

[2]Laboratoire de Chrono-Environnement, UMR UFC/CNRS 6249 USC INRA, Université de Franche-Comté, 25030 Besançon cedex, France

[3]Institute of Biochemistry and Biophysics, Polish Academy of Sciences, ul. Pawinskiego 5a, 02-106 Warsaw, Poland

[4]UPSP PROXISS, ENESAD, 26 Boulevard Dr Petitjean, BP 87999, 21079 Dijon cedex, France

[#]Joint first authors

Corresponding author: David Wendehenne

Tel: +33 380 69 37 22; Fax: +33 380 69 32 26; Email: wendehen@dijon.inra.fr

Abstract:

Nitric oxide (NO) is a gaseous free radical recognized as a ubiquitous signal transducer that contributes to various biological processes in animals. It exerts most of its effects by regulating the activities of various proteins including Ca^{2+} channels, protein kinases and transcription factors. In plants, studies conducted over the past ten years revealed that NO also functions as an endogenous mediator in diverse physiological processes ranging from root development to stomatal closure. Its biological role as an intracellular plant messenger molecule, however, remains poorly understood. Here, we review the molecular basis of NO signaling in animals and discuss current knowledge of NO signaling in plants, focusing on its interplay with Ca^{2+}, protein kinases and reactive oxygen species which are well established as widespread key regulators of signal transduction.

Keywords:

Calcium, cell death, nitric oxide, protein kinases, reactive oxygen species, signaling

Nitric oxide (NO) is a noxious free radical gas which, in the late 1980s, was discovered to exist physiologically in mammalian systems. This discovery offered fresh perspectives on main processes including neurotransmission, immunity and relaxation of vascular smooth muscles (Schmidt and Walter, 1994). Notably, the idea that a simple gas could act as a messenger revolutionized researcher understanding of signal transduction. Recently, NO was also shown to mediate diverse plant physiological processes such as germination, root growth, flowering, stomatal closure and resistance to biotic as well as abiotic stresses (see reviews by Lamattina *et al*., 2003; Delledonne, 2005; Besson-Bard *et al*., 2008a; Wilson *et al*., 2008). Although evidences supporting NO as a plant physiological mediator are still growing, its functions at the molecular level remain poorly understood and, in some examples, are subjected to controversies. Research conducted over the past years has revealed that NO mediates part of its action in a concerted way with the second messenger Ca^{2+}, protein kinases and reactive oxygen species (ROS). The interplays between these molecules operate in cells challenged by biotic and abiotic stresses and modulate various cellular responses including gene expression and cell death. This review introduces the basic concepts of NO signaling in animals and discuss the mechanisms through which NO exerts its signaling activities in plants with a particular emphasis on Ca^{2+}, protein kinases and ROS signaling.

Basic concepts of NO signaling in animals

The field of research dedicated to NO signaling in animals has been extraordinary fruitful in the past two decades and has led scientists to introduce new concepts of signal transduction. NO is derived from the amino acid L-arginine by the enzymatic activity of nitric oxide synthase (NOS). Once produced, NO acts predominantly *via* the post-translational modifications of proteins. Three main processes have been described: *S*-nitrosylation, metal nitrosylation and tyrosine nitration. Well over a hundred proteins susceptible to these NO-dependent post-translational modifications and involved in all major cellular activities have been identified. In this section, we describe the principles of *S*-nitrosylation, metal nitrosylation and tyrosine nitration and discuss how these post-translational protein modifications influence Ca^{2+} and protein kinase signaling. Understanding these signaling concepts should facilitate a comprehensive analysis of the way NO acts as a signal in plants.

Metal Nitrosylation

As a radical, NO is capable of donating electrons and therefore reacts with transition metals. Covalent interaction of NO with the centers of iron-sulfur clusters, heme and zinc-finger proteins leads to an increase or a decline in protein activity. Amongst the proteins regulated through metal nitrosylation, a well-studied target for NO is soluble guanylate cyclase (sGC; Denninger and Marletta, 1999). sGC catalyses the conversion of GTP to pyrophosphate and 3',5'-cyclic GMP (cGMP), a well-defined second messenger. The interaction of NO with the sGC heme leads to the opening of the bond between ferrous iron and histidine 105 of the enzyme, thus triggering a conformational change that increases the catalysis of cGMP synthesis by several hundred-fold (Cary *et al.*, 2006; Roy and Garthwaite, 2006). Once produced, cGMP binds to target proteins: cGMP-dependent protein kinases (PKGs), cyclic-nucleotide-gated channels (cCNGCs) and cyclic-nucleotide phosphodiesterases, resulting in cell-specific downstream outputs (Beck *et al.*, 1999). Examples of physiological responses regulated through NO/cGMP signaling include neurotransmission, development, smooth muscle relaxation and blood pressure regulation (Denninger and Marletta, 1999; Krumenacker *et al.*, 2004).

S-nitrosylation

S-nitrosylation corresponds to the covalent modification of cysteine sulfurs of proteins by NO (or its derivatives) to form *S*-nitrosothiols (Stamler *et al.*, 2001; Hess *et al.*, 2005). It is not yet clear how NO *S*-nitrosylates target proteins. Candidate mechanisms include the electrophilic attack of the nitrosonium cation (NO^+, resulting from NO auto-oxidation) on thiolate, direct interaction of NO with thiolate in the presence of electron acceptors such as NAD^+ and complex chemical processes involving nitroxyl anions (NO^-, resulting from NO auto-reduction or dinitrogen trioxide decomposition) (Gow *et al.*, 1997; Hanafy *et al.*, 2001; Foster and Stamler, 2004). Interestingly, primary peptide sequences for motifs that might facilitate *S*-nitrosylation have been described, consisting of acidic/basic motifs, as well as hydrophobic motifs surrounding the cysteine residue (Hess *et al.*, 2005; Greco *et al.*, 2006). Similarly to metal nitrosylation, *S*-nitrosylation is a reversible form of post-translational modification. De-*S*-nitrosylation occurs chemically without the help of enzymes or

enzymatically through thioredoxin and thoredoxin reductase (Jaffrey *et al.*, 2001; Benhar *et al.*, 2008).

Tyrosine nitration

Tyrosine nitration is mediated by two main NO-derived species including peroxynitrite ($ONOO^-$), resulting from the fast reaction between NO and ROS such as superoxyde ($O_2^{\cdot -}$), and nitroso-peroxocarboxylate ($ONOOCO_2^-$), an adduct formed following the reaction between $ONOO^-$ and CO_2 at a physiological concentration (Radi, 2004). Nitration occurs in one of the two equivalent carbon atoms in the ortho position (with respect to the hydroxyl group) of the phenolic ring of tyrosine residues and leads to protein 3-nitrotyrosine (3-NO_2-Tyr) (Radi, 2004). 3-NO_2-Tyr was first related to NO-dependent oxidative stress occurring during inflammatory diseases such as asthma (Schopfer *et al.*, 2003). Indeed, tyrosine nitration is usually associated with loss of protein functions and target proteins include Mn superoxide dismutase, cytochrome P450, tyrosine hydroxylase, glutamine synthase, glutathione reductase, actin and other cytoskeleton-related proteins (Greenacre and Ischiropoulos, 2001; Gow *et al.*, 2004). It is generally assumed that this process may be irreversible and increase the susceptibility of proteins to degradation by the 20S proteasome (Mannick and Schonhoff, 2002). However, the description of denitrase activities reversing protein nitration in several mammalian tissues suggests that tyrosine nitration might also be a reversible process (Gorg *et al.*, 2007). This latter finding opens the possibility that the formation of 3-NO_2-Tyr may play a role in signal transduction. Regarding this aspect, the relationship with protein tyrosine phosphorylation is particularly noteworthy. Indeed, according to several studies, the importance of tyrosine nitration on cell signaling would lie essentially in the inhibition of tyrosine residues to undergo phosphorylation and/or in the inhibition of phosphatases that allows protein kinases to become dominantly activated (Minetti *et al.*, 2002). A first mechanism has been proposed to explain the activation of tyrosine kinases c-Src by $ONOO^-$: *in vitro* nitration of a C-terminal tyrosine residue could prevent its phosphorylation which normally helps c-Src folding into an inactive conformation (Klotz *et al.*, 2002). At present, however, it is unclear if this process can occur *in vivo*.

Interplays between NO and Ca^{2+}

Currently, NO is recognized as one of the key messengers governing the overall control of Ca^{2+} homeostasis, and almost all types of Ca^{2+} channels and transporters are under its control. The effects of NO on Ca^{2+} channel and transporter activities can be divided into two mechanisms of action: a cGMP-dependent one and a cGMP-independent one. The molecular mechanisms underlying the cGMP-dependent pathway are complex, and at least three processes have been reported. First, cGMP could directly activate CNGCs by virtue of their cyclic-nucleotide-binding sites, leading to an enrichment of cytosolic free Ca^{2+} concentrations ($[Ca^{2+}]_{cyt}$) (Hanafy *et al.*, 2001; Ahern *et al.*, 2002). Second, the effects of cGMP could be mediated via the activation of PKGs (Clementi and Meldolesi, 1997; Clementi, 1998; Ahern *et al.*, 2002). PKGs have distinct effects on intracellular Ca^{2+}, increasing or decreasing $[Ca^{2+}]_{cyt}$, depending on the target channel, the stimuli and cell types. For example, in hepatocytes, phosphorylation of the inositol 1,4,5-triphosphate (IP_3) receptor by PKGs potentiates IP_3-dependent Ca^{2+} release, whereas an opposite effect is observed in smooth muscle (Clementi, 1998; Murthy and Makhlouf, 1998). Besides the IP_3 receptor, Ca^{2+}-permeable channels and Ca^{2+} transporters whose activities appear to be modulated by PKGs include voltage-dependent Ca^{2+} channels (L-, N-, P/Q- and T-types), store-operated Ca^{2+} channels (SOCCs), mechano-sensitive Ca^{2+}-permeable non-selective cation channels (MS-NSCs), the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) and the plasma membrane Ca^{2+} pump (PMCA) (Clementi, 1998; Wang *et al.*, 2000; Chen *et al.*, 2002; Yao and Huang, 2003; Grassi *et al.*, 2004). The biochemical steps downstream of PKGs that are responsible for the modulation of these channels and transporters have not been completely clarified. Third, to add further complexity to these scenarios, PKG activation has been found to be a crucial step in NO-induced cyclic ADP-ribose (cADPR) synthesis (Willmott *et al.*, 1996; Reyes-Harde *et al.*, 1999; Leckie *et al.*, 2003). cADPR is synthesized from its precursor NAD^+ by ADP-ribosyl cyclase which might be activated through PKG-induced phosphorylation. cADPR is a Ca^{2+} mobilizing second messenger which promotes Ca^{2+} release from endoplasmic reticulum in a wide variety of animal cells *via* the activation of the ryanodine receptors (RYRs) (Fliegert *et al.*, 2007). The cGMP/PKG/cADPR cascade is now recognized as a fundamental mechanism through which NO contributes to the generation and propagation of Ca^{2+} signals in various physiological processes including the induction of hippocampal long-term depression and fertilization in echinoderms (Willmott *et al.*, 1996; Reyes-Harde *et al.*, 1999; Leckie *et al.*, 2003).

The cGMP-independent action of NO on Ca^{2+} homeostasis operates through the direct *S*-nitrosylation of Ca^{2+} channels and transporters. Voltage-dependent Ca^{2+} channels, RYRs,

N-methyl-D-aspartate (NMDA) receptors, transient receptor potential channels (TRPC) and CNGCs were shown to be reversibly *S*-nitrosylated, with activation or inhibition as a consequence (Broillet, 2000; Stamler *et al.*, 2001, Yoshida *et al.*, 2006; Tjong *et al.*, 2007). For instance, the skeletal muscle RyR1 (one of the three isoforms of RYRs) consists of four homologous 565 kDa subunits containing 100 cysteine residues. In the native protein, 50 of these residues appear to be in a reduced state (Aracena-Parks *et al.*, 2006). Remarkably, submicromolar NO concentrations were shown to activate RYR1 by *S*-nitrosylation of a single cysteine (Cys 3635), this reaction occurring only at low (e.g. physiological) pO_2 but not ambient pO_2 (Sun *et al.*, 2003). This specific *S*-nitrosylation reverses RYR1 inhibition by Ca^{2+}/Calmodulin (CaM) and may contribute to enhanced RYR1 activity. Interestingly, Cys 3635 can also be *S*-glutathionylated, suggesting that competition between *S*-nitrosylation and *S*-glutathionylation on Cys 3635 may occur in physiological processes (Aracena-Parks *et al.*, 2006). Another remarkable example of the influence of *S*-nitrosylation on Ca^{2+} channel activities concern TRPC5, one of the seven TRPC homologs in human. This plasma membrane Ca^{2+} channel was shown to induce Ca^{2+} entry into human embryonic kidney cells in response to NO released by several NO donors (Yoshida *et al.*, 2006). The molecular mechanism underlying NO-dependent TRPC5 activation may involve the nucleophilic attack of nitrosylated Cys 553 by the free sulfydryl group of Cys 558, thus leading to the formation of a disulfide bond between both cysteine residues. The disulfide bond might stabilize the open state of the channel.

The existence of both cGMP-mediated and direct *S*-nitrosylation pathways expands and enriches the possibilities for NO to modulate Ca^{2+}-dependent signaling processes including gene expression (Peunova and Enikolopov, 1993). Furthermore, because NO production by NOS requires an increase in $[Ca^{2+}]_{cyt}$, the ability for NO to attenuate Ca^{2+} influx by inhibiting some types of Ca^{2+} channels and/or to initiate cytosolic free Ca^{2+} removal by activation of SERCA and/or PMCA helps to protect cells from the deleterious effect of NO. The pathophysiological relevance of these processes is outlined in several examples such as the modulation of neuronal excitability or hypertension but has probably paved the way for new roles in normal and disturbed cell functions. From a mechanical point of view, the plasticity of the NO/Ca^{2+} pathways is particularly intriguing when both pathways act on the same channels. Several studies have provided support that the cGMP-dependent pathway generally occurs at low levels of NO whereas *S*-nitrosylation requires higher levels of NO and tends to proceed with slower kinetics than cGMP-induced actions (Denninger and Marletta,

1999; Hanafy *et al.*, 2001). However, this subject is still controversial and the issue is far from settled (Stamler *et al.*, 2001; Hess *et al.*, 2005).

NO signaling in plants

During the last decade, NO has been recognized as a versatile player in diverse plant physiological processes. Several routes for NO synthesis have been described: non-enzymatic as well as enzymatic pathways involving nitrate reductase and putative NOS-like enzymes (Kaiser and Huber, 2001; Crawford, 2006; Corpas *et al.*, 2006; Besson-Bard *et al.*, 2008a, 2008b and 2008c; Wilson *et al.*, 2008). A major and still opened question concerns the molecular mechanisms of its signaling action. More than hundred proteins have been asserted to undergo regulation by *S*-nitrosylation and metal nitrosylation. Similarly, numerous genes up- and/or down-regulated in response to artificially produced NO have been identified based on microarray analyses. However, with few exceptions, the physiological significance of these post-transcriptional and post-translational modifications remains to be established. Parallel to these approaches, over the last years, evidence gathered from a number of studies has indicated that NO mediates part of its effects through the mobilization of free Ca^{2+}, *via* the modulation of protein kinases activities and by interacting with ROS. The aim of this section is to concentrate on the interplay between NO, Ca^{2+} and ROS and to describe what is known thus far concerning the physiological impact of the cross-talk between these signaling components.

Interplays between NO and Ca^{2+}

A large number of signals, including plant hormones, light, biotic as well as abiotic stresses cause transient and specific changes in intracellular [Ca^{2+}]. In the recent years, it has become increasingly appreciated that the signaling components that govern these changes include NO. Furthermore, because NO production is under the control of intracellular Ca^{2+} fluctuations, NO might also act as a Ca^{2+} sensor contributing to decoding the intracellular Ca^{2+} changes in plants.

NO acts as a Ca^{2+}-mobilizing messenger

The first conclusive evidence implicating NO as a Ca^{2+} mobilizing messenger in plant cells came from studies exploring the ability of NO donors to induce increases in intracellular [Ca^{2+}]. Using Ca^{2+}-sensitive dye fura 2 fluorescence ratio imaging, Garcia-Mata *et al.* (2003) showed that treating *Vicia faba* guard cells by the NO donor *S*-nitroso-*N*-acetyl-penacillamine (SNAP) induced stomatal closure by promoting an increase in intracellular [Ca^{2+}]. The ability of exogenous NO to induce a rise of intracellular [Ca^{2+}] was further supported by the finding that the NO donor diethylamine NONOate (DEA/NO) triggers a transient rise of $[Ca^{2+}]_{cyt}$ in transgenic *Nicotiana plumbaginifolia* cell suspensions expressing the Ca^{2+}-reporter apo-aequorin (Lamotte *et al.*, 2004 and 2006). By contrast, the same NO donor did not induce any change in nuclear free Ca^{2+} concentration ($[Ca^{2+}]_{nuc}$) (Lecourieux *et al.*, 2005) suggesting that NO effects on Ca^{2+} homeostasis is restricted to specific cellular compartments.

The influence of NO on the cellular [Ca^{2+}] in physiological contexts was highlighted by the demonstration that NO scavengers and mammalian NOS inhibitors reduced stimulus-induced rises in $[Ca^{2+}]_{cyt}$. Notably, Lamotte *et al.* (2004) showed that the NO scavenger cPTIO and NOS inhibitors reduced the $[Ca^{2+}]_{cyt}$ increases induced in *N. plumbaginifolia* cells by the proteinaceous elicitor cryptogein secreted by the oomycete *Phytophthora cryptogea*. These pharmacological agents did not reduce the cryptogein-triggered $[Ca^{2+}]_{nuc}$ rises, confirming the observation made using NO donors (see above; Lecourieux *et al.*, 2005). Similar effects of NO were reported in grapevine cell suspensions exposed to the elicitor endopolygalacturonase 1 from *Botrytis cinerea* (Vandelle *et al.*, 2006). Another example emerges from studies investigating NO function in plant cells exposed to hyper-osmotic stress. Under this condition, addition of the NO scavenger cPTIO reduced the increase in $[Ca^{2+}]_{cyt}$ in *N. plumbaginifolia* cell suspensions expressing the calcium reporter apo-aequorin, highlighting again the role of NO in $[Ca^{2+}]_{cyt}$ elevations (Gould *et al.*, 2003; Lamotte *et al.*, 2006).

Underlying mechanisms

Research has been directed towards identifying the mechanisms through which NO triggers changes in intracellular [Ca^{2+}]. Pharmacological analyses of [Ca^{2+}] variations have indicated that NO might activate both plasma membrane and intracellular Ca^{2+}-permeable channels (Garcia-Mata *et al.*, 2003; Lamotte *et al.*, 2004 and 2006; Lecourieux *et al.*, 2005; Vandelle *et al.*, 2006). Whereas the putative identity of the NO-sensitive plasma membrane Ca^{2+}-permeable channels remains unknown, several lines of evidence have suggested that NO might target RYR-like channels. Indeed, the increase in $[Ca^{2+}]_{cyt}$ of *N. plumbaginifolia* cells

treated by the NO donor DEA/NO was sensitive to ruthenium red (RR), an inhibitor of mammalian RYRs (Lamotte *et al.*, 2004). A similar inhibitory effect was obtained using the cADPR antagonist 8Br-cADPR, designing cADPR as possible intermediates of the NO signal leading to changes in intracellular [Ca^{2+}]. According to Garcia Mata *et al.* (2003), cADPR might function together with cGMP as reported in animals (Willmott *et al.*, 1996; Reyes-Harde *et al.*, 1999). Indeed, they showed that ryanodine, an antagonist of RYR as well as ODQ (1-H-(1,2,4)-oxadiazole-(4,3-a)quinolxalin-1-one), an inhibitor of sGC, were able to suppress SNAP-mediated increase in intracellular [Ca^{2+}] in *V. faba* guard cells. The authors provided several arguments indicating that a similar NO/cGMP/cADPR/Ca^{2+} pathway might occur in response to abscisic acid (ABA).

Besides cADPR and cGMP, evidences that NO could also contribute to $[Ca^{2+}]_{cyt}$ increases *via* phosphorylation events were provided. Indeed, protein kinase inhibitors efficiently suppress NO donors-triggered elevation in $[Ca^{2+}]_{cyt}$ in *V. faba* guard cells and *N. plumbaginifolia* cells (Sokolovski *et al.*, 2005; Lamotte *et al.*, 2006), indicating that the signaling cascades relaying NO and Ca^{2+}-permeable channels could involve protein kinases, besides or together with cADPR. At present, only one protein kinase candidate, named NtOSAK (*Nicotiana tabacum* Osmotic Stress-activated protein Kinase) has been identified (Lamotte *et al*., 2006). This aspect is discussed farther. Finally, it should be specified that several arguments pointing out the involvement of NO in stimulus-induced plasma membrane depolarization have been reported (Lamotte *et al*., 2006; Vandelle *et al*., 2006). Such NO-dependent changes of the plasma membrane potential might modulate the activity of plasma membrane Ca^{2+}-permeable channels. The mechanisms underlying this effect are poorly understood.

Impacts of the NO/Ca^{2+} pathways

The first conclusive evidence of the biological significance of a NO/Ca^{2+} pathway came from studies in which the function of NO in plant defence responses was investigated (Durner *et al*., 1998; Klessig *et al*., 2000). Using *N. tabacum* plants and suspension cells treated with a recombinant mammalian NOS or NO donors, respectively, it was shown that NO was able to mediate the expression of the defence-related genes *PR* (*pathogenesis related-1*)-1 and *PAL* (*phenylalanine ammonia lyase*) through cGMP and/or cADPR. In addition to gene expression, compelling evidence suggests a role for the interplay between NO and Ca^{2+} on microorganism-triggered hypersensitive response (HR). In this context, NO appears to act as a Ca^{2+} sensor contributing to decode the intracellular Ca^{2+} changes in plants leading to cell

death. Both pharmacological and genetic experimental data support this concept (Delledonne *et al.*, 1998; Lamotte *et al.*, 2004; Ali *et al.*, 2007). For instance, in cryptogein-elicited tobacco cell suspensions, NO production is stimulated by an influx of extracellular Ca^{2+} (Lamotte *et al*., 2004). In turn, NO partly contributes to the elicitor-triggered cell death. The plasma membrane cyclic nucleotide-gated Ca^{2+}-permeable channel CNGC2 was recently identified as one of the putative key component of this pathway in *Arabidopsis thaliana* (Ali *et al.*, 2007). Accordingly, the HR normally suppressed in the *A. thaliana dnd1* (*defence no death 1*) mutant impaired in CNGC2 expression, was shown to be partially restored by the NO donor sodium nitroprusside (SNP). Further research is needed to understand how NO, the production of which is stimulated by an influx of Ca^{2+}, can contribute to HR. Because, as discussed previously, NO also amplifies the mobilization of free Ca^{2+}, it is possible that the NO-dependent rise of intracellular [Ca^{2+}] facilitates cellular Ca^{2+} overload which, in turn, could cause cytotoxicity and could trigger cell death. Besides mediating defence responses, the NO/Ca^{2+} pathways might influence diverse cellular processes such as ABA-induced stomatal closing or auxin-mediated adventitious root formation (Garcia-Mata *et al*., 2003; Lamattina *et al*., 2003; Desikan *et al*., 2004).

Interplays between NO and protein kinases

Upon receiving a signal, cells often utilize multiple protein kinase cascades to transduce and amplify the information. Protein phosphorylation and dephosphorylation are very common intracellular signaling modes. Kinases and phosphatases regulate a wide range of cellular processes such as enzyme activation, assembly of macromolecules, protein localization and degradation. In animals, NO has been described to modify the activity of protein kinases involved in signal transduction, such as mitogen activated protein kinase (MAPK) cascades, Janus kinases or protein kinase C (Beck *et al*., 1999). Also, the activity of primary metabolism related kinases, for instance pyruvate kinase, were identified to be modified by *S*-nitrosylation (Gao *et al*., 2005).

It is presently known that serine/threonine protein kinases play a crucial role in the transduction of various extra- and intracellular signals in plants (Mishra *et al.*, 2006). However, although the identification of NO-modulated protein kinases is a major issue in the understanding of NO-dependent signal transduction, only few of them have been identified and studied. Moreover, these observations have been carried out using, for most part of them, artificially generated NO from NO donors, and not during a physiological plant process.

NO modulates MAPK activities

In all Eukaryotes, MAPK pathways serve as highly conserved central regulators of growth, death, differentiation, proliferation and stress responses (Samaj *et al.*, 2004; Qi and Elion, 2005). MAPKs form the terminal components of the MAPK cascades (MAPKKK→MAPKK→MAPK). MAPKs are activated by MAPK kinases (MAPKKs/MEKs) *via* dual phosphorylation of conserved threonine and tyrosine residues in the motif TxY located in the activation loop. Some evidence shows that in plants NO also contributes to the activation of MAPK cascades.

Kumar and Klessig (2000) found a MAPK activated by NO in tobacco leaves and cell suspensions. Injection of tobacco leaves with recombinant rat neuronal NOS, together with its cofactors and substrate transiently activated a 48-kDa protein kinase phosphorylating MBP (myelin basic protein), an artificial MAPK substrate. Using specific anti-SIPK antibody in immuno-complex kinase activity assay, they identified this kinase as Salicylic Acid (SA)-Induced Protein Kinase (SIPK). The NO donors S-nitroso-L-glutathione (GSNO), DEA/NO and SNAP also transiently activated SIPK in tobacco cell suspension cultures (Kumar and Klessig, 2000; Besson-Bard *et al.*, 2008b). Depending on the NO-generating system, this activation was shown to be SA-dependent or SA-independent.

Although SIPK is the first and the only NO-dependent MAPK identified to date, there are more data indicating influence of NO on MAPK pathways. Working with *A. thaliana* shoots, Capone *et al*. (2004) demonstrated that brief oxidative or nitrosative stresses in the roots, using respectively H_2O_2 and the NO donor SNP, triggered the activation of a 38-kDa protein kinase able to phosphorylate MBP. It was confirmed that this kinase belongs to the MAPK family by using antibodies raised against the active (phosphorylated) form of a mammalian p38 MAPK, but no direct relation between this activation and NO production has been clearly demonstrated. Another example of NO ability to activate MAPK was provided by Clarke *et al*. (2000). The authors reported that a 47-kDa protein kinase, able to phosphorylate MBP, is activated within 5 minutes in response to the NO donor Roussin's black salt (RBS) in *A. thaliana* cell suspensions. A role for the 47-kDa protein kinase in mediating NO-induced cell death was tentatively assigned. However, pharmacological inhibition of this MAPK was inefficient in reducing cell death, leading opened the question of the cellular impact of the activation of this MAPK by NO.

More recently, a functional link between NO and MAPKs has been established in ABA signaling in mesophyll cells of maize leaves (Zhang *et al*., 2007). Using

pharmacological approach, a linear interplay of these signaling components has been demonstrated: ABA treatment induces H_2O_2 production acting upstream NO synthesis. In turn, NO favours the activation of a 46-kDa MAPK. Induction of this MAPK results in an enhancement of the expression of genes encoding antioxidant proteins such as catalase, superoxide dismutase, gluthatione reductase or ascorbate peroxidase, thus improving the total antioxidative activity of the cells. This cascade of reactions could be triggered in response to stresses such as water-stress, thus highlighting a key role for NO in controlling MAPK involved in the plant adaptive response to abiotic stresses.

Finally, several lines of evidence suggest that NO and MAPKs act together in the auxin transduction pathway leading to adventitious root formation. More precisely, pharmacological-based experiments designed NO as a key regulator of an auxin-induced 48-kDa MAPK sensitive to the MAPK inhibitor PD098059 (Pagnussat *et al*., 2004). Cucumber explants co-treated with a NO donor and PD098059 showed a significant reduction in root length and root number, demonstrating firstly that NO is required for the activation of the 48-kDa MAPK, and secondly that this activation is essential for adventitious root formation. Interestingly, addition of a sGC inhibitor was not able to prevent the NO-dependent activation of the 48-kDa MAPK activation. This latter result suggested that this MAPK might be part of a NO-dependent/cGMP-independent signaling pathway which parallels a previously characterized NO/cGMP-dependent signaling cascade also acting in auxin-induced adventitious root formation (Pagnussat *et al*., 2003).

NO and Ca^{2+}-dependent protein kinases

Support for the hypothesis that NO promotes the activation of Ca^{2+}-dependent protein kinases (CDPKs) came through biochemical and pharmacological approaches that showed the ability of SNP and auxin to induce the activation of a 50-kDa protein kinase in a Ca^{2+}-dependent manner in cucumber hypocotyls (Lanteri *et al*., 2006). The auxin- or SNP-triggered activation of the 50-kDa protein kinase was also reduced by CaM antagonists including trifluoperazine dihydrochloride (TFP) and N-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide hydrochloride (W-7). These results let to the assumption that the 50-kDa protein kinase may contain CaM-like Ca^{2+}-binding domain, a structural feature of CDPKs. However, it should be noticed that both TFP and W-7 are not specific inhibitors of CDPKs. Indeed, these compounds also affect the binding of Ca^{2+} to proteins such as CaM and calcineurin B-like proteins (Anil and Rao, 2000) which normally regulate the activity, and

therefore the function, of a variety of target proteins including protein kinases (e.g. CcaMKs, CaMKs, SnRKs3; Hrabak *et al*., 2003).

At a physiological level, the CaM antagonists TFP and W-7 were shown to negatively affect NO- or auxin-induced adventitious root formation in cucumber, suggesting the involvement of the 50-kDa protein kinase in this process (Lanteri *et al*., 2006). Interestingly, in contrast to the NO-dependent 48-kDa MAPK described above, the activity of the 50-kDa putative CDPK triggered by SNP or auxin was inhibited by sGC inhibitors, suggesting that this protein kinase is part of the NO/cGMP-dependent pathway leading to adventitious root formation (Pagnussat *et al*., 2003). Because the activity of the 50-kDa cucumber CDPK was detected at the earlier stages of adventitious root formation, it was proposed that this NO-dependent protein kinase can be involved in cell dedifferentiation, division and/or differentiation (Lanteri *et al*., 2006).

NO and SnRKs

Plant SNF1 (sucrose nonfermenting 1)-related protein kinases (SnRKs) are classified into three subfamilies: SnRK1, SnRK2, and SnRK3. Available evidence indicate that SnRK1 might play an important role in the regulation of global metabolism, the disturbance of which might lead to developmental or adaptation defects (for reviews see Halford and Hardie, 1998; Halford *et al*., 2003; Hrabak *et al*., 2003). The SnRK2 and SnRK3 subfamilies are specific to plants and are involved in environmental stress signaling (for reviews see Hrabak *et al*., 2003; Boudsocq and Lauriere, 2005).

First evidence that NO modulates the activity of SnRKs was provided by Lamotte *et al*. (2006). These authors showed that application of the NO donor DEA/NO to tobacco cell suspensions resulted in a fast and transient activation of a 42-kDa protein kinase phosphorylating MBP and histone IIIS, another protein kinase substrate. Using specific antibodies in immuno-complex activity assay, this 42-kDa protein kinase was identified as NtOSAK, a member of the SnRK2 family (Kelner *et al*., 2004). Similarly to other SnRK2 members in *A. thaliana* (Droillard *et al*., 2002; Boudsocq *et al*., 2004) and rice (Kobayashi *et al*., 2004), NtOSAK is activated within minutes in response to hyperosmotic stress (Mikolajczyk *et al*., 2000), a process which leads to a rapid increase in NO synthesis (Gould *et al*., 2003). Importantly, NtOSAK activation in response to osmotic stress was abolished by the NO scavenger cPTIO, highlighting the ability of NO in promoting SnRK2 activation during physiological processes.

These finding might be of general importance because it is consistent with the central roles of both NO and SnRK2s in the regulation of stomatal closure as well as defence responses. This assumption is exemplified by the involvement of NO and the *A. thaliana* SnRK2 protein kinase OST1 (open stomata 1) in common pathways. Indeed, OST1 was found to mediate the regulation of stomatal closure by ABA, a function also assigned to NO (Mustilli *et al*., 2002). Similarly, the pathogen-associated molecular pattern (PAMP) flagellin 22 and lipopolysaccharide (LPS) were shown to trigger stomatal closure through a NO- and OST1-dependent signaling cascade (Melotto *et al*., 2006). Although a mechanistic connection between NO and OST1 remains to be established, these data further support the hypothesis that NO and SnRK2 protein kinases act together in the plant adaptive responses to biotic as well as abiotic stresses.

Interplays between NO and ROS

Impacts of the NO/ROS balance in HR

The interplay between ROS and NO has long been recognised in the animal field (Curtin *et al*., 2002). In fact, many of the NO derived responses are believe to stem from the reaction between NO and ROS to form reactive nitrogen species (RNS) such as $ONOO^-$. In contrast to ROS, NO is exclusively produced by specific enzymes in animal cells (Turpaev and Litvinov, 2004). Conversely, plants can produce NO through a number of pathways: either enzymatic or not (Kaiser and Huber, 2001; Corpas *et al.*, 2006; Crawford *et al.*, 2006; Besson-Bard *et al.*, 2008a, 2008b and 2008c; Wilson *et al*., 2008). An interaction between both molecules during the HR was originally suggested by Delledonne *et al*. (1998 and 2001). The authors observed a strong NO burst accompanied by ROS generation following inoculation of soybean cell cultures with avirulent bacteria. However, the up-regulation of NO following this bacterial infection was not sufficient to activate the HR-cell death and, rather unexpectively, $ONOO^-$ was not responsible for cell death. Thus, although $O_2\cdot^-$ is not directly involved in this response, its conversion to H_2O_2 by superoxyde dismutase is critical for the H_2O_2/NO signaling pathway.

De Pinto *et al*. (2002 and 2006) also demonstrated that the NO/ROS couple is necessary for programmed cell death (PCD) in *N. tabacum* cv. BY-2 cells and a strong spatio-temporal correlation was reported between ROS and NO production during powdery mildew-

dependent HR in barley (Mur *et al*., 2008). The HR elicited by *Pseudomonas syringae* pv. *phaseolicola* and pv. *tomato* harbouring the *avrRpm1* gene in tobacco and *A. thaliana* was preceded by an NO peak followed immediately by an H_2O_2 burst (Mur *et al*., 2005). Similarly, inoculation of *A. thaliana* cell cultures with *P. syringae* pv. *maculicola* carrying the *avrRpm1* avirulence gene resulted in a rapid and sustained NO increase whereas the increase production of H_2O_2 was delayed (Clarke *et al*., 2000). Thus, although some discrepancies exist in the literature concerning the cooperation between NO and H_2O_2, the NO/H_2O_2 balance still seems crucial for many HR-dependent cell death events.

The question may thus arise as to how do cellular NO and H_2O_2 interact during the HR. A mechanistic answer was recently provided by Romero-Puertas *et al*. (2007). Employing a proteomic strategy based on the biotin-switch assay, the authors identified several proteins in which *S*-nitrosylation level is increased in *A. thaliana* leaves challenged by the incompatible pathogen *P. syringae*. The authors focused their attention on peroxiredoxin II E (PrxII E), a member of the peroxiredoxin family which catalyses the reduction of H_2O_2 but also $ONOO^-$, depending on the isoforms. Extensive biochemical and genetic approaches indicate that *S*-nitrosylation of PrxII E inhibits its capacity to detoxify $ONOO^-$. Based on these data, an interesting model was proposed in which *S*-nitrosylation of PrxII E impairs its peroxynitrite reductase activity, thus leading to an increased level of tyrosine nitration, a hallmark of NO/ROS-dependent oxidative stress.

Candidate sites of interaction between NO and ROS during the HR

In animals, mitochondria play a central role in PCD by releasing cytochrome *c* and activating caspases, and there is growing belief that the intracellular redox status is critical in mitochondria-dependent cell death in animals (Kowaltowski *et al.*, 2001). In particular, the interaction between mitochondrial cytochrome c and NO constitutes an important signaling pathway for the controlled production of H_2O_2 (Brookes *et al*., 2002). In plants, mitochondria have recently been identified as key players of cell redox homeostasis and signaling (Noctor *et al*., 2007), as well as important integrators of PCD (Jones, 2000; Swidzinski *et al.*, 2002 and 2004; Lam, 2004). Transgenic tobacco cells lacking the alternative oxidase show enhanced susceptibility to various cell death inducers, including H_2O_2 (Robson and Vanlerberghe 2002; Vanlerberghe *et al.*, 2002) and H_2O_2-driven cell death occurs through a mitochondria-dependent pathway (Mur *et al.*, 2008). Interestingly, mitochondria are also considered as potential sites of NO action. Notably, although oxygen consumption *via* the

cytochrome pathway is inhibited by NO in isolated soybean cotyledons (Millar and Day, 1996) and carrot cell suspensions (Zottini *et al*., 2002), in both cases the cyanide insensitive alternative oxidase is not significantly affected. Similarly, Yamasaki *et al*. (2001) found that the alternative pathway is resistant to NO in plant mitochondria isolated from mung bean. Thus, these data suggest that the NO effect on the respiratory pathway may play some role in maintaining mitochondrial homeostasis by limiting ROS release. Further support for this comes from the fact that mitochondria can support nitrite-dependent NO synthesis (Planchet *et al.*, 2005) and that AtNOA1 (Nitric Oxide Associated 1), an enzyme initially thought to display NOS activity, is targeted to the mitochondria (Guo and Crawford, 2005). Considering the hydrophobic and diffusible nature of NO, these data provide additional support for a potential interaction between NO and ROS in the mitochondria or its vicinity, thus potentially participating in mitochondria derived cell death signals.

The chloroplast has also recently been put forward as a critical player in the development of the HR under light (Zeier *et al*., 2004; Montillet *et al*., 2005; Mur *et al*., 2008). NO can inhibit chloroplast electron transport in a reversible manner (Takahashi and Yamasaki, 2002) and chloroplasts have also been identified as potential participant in NO synthesis and $ONOO^-$ production in plants (Gould *et al*., 2003; Jasid *et al*., 2006). In fact, a strong correlation between $ONOO^-$ and the presence of oxidatively modified proteins in both the stroma and the thylakoids was observed in soybean chloroplasts (Jasid *et al.*, 2006). Furthermore, it was suggested that $ONOO^-$ interacts with non-heme Fe^{2+} leading to PSII inhibition on the acceptor site (Gonzalez-Perez *et al*., 2008). Thus, *in situ* production of NO in the chloroplasts could play a protective role in preventing oxidation of chloroplastic lipids and proteins but alternatively, the reaction between $O_2\cdot^-$ and NO could lead to $ONOO^-$ production which could be responsible for the impairment of the photosynthetic machinery. Thus, the different HR cell death phenotypes observed in the light or in the dark during pathogen infection (Montillet *et al*., 2005) may therefore depend on the effect of NO on chloroplastic homeostasis but also on the release of ROS by the chloroplast and their interaction with NO.

Finally, both ROS and NO can also be produced in the peroxisomes. In fact, peroxisomes are not only a major site of $O_2\cdot^-$ and H_2O_2 production (Del Rio *et al*., 2002) but they have also been proposed a major site of NO synthesis (Corpas *et al*., 2001). In a recent study, the possible interaction between NO and peroxisomal H_2O_2 production on gene regulation was analysed in transgenic catalase antisense tobacco plants (Zago *et al*., 2006). The different phenotypes obtained under various concentrations of H_2O_2 and NO clearly

supported the idea that a tight balance between both molecules is necessary for HR-type cell death. Furthermore, this cDNA-AFLP analysis demonstrated that only 16 differentially expressed transcripts required both NO and H_2O_2. In contrast, 152 genes could be modulated by either NO or H_2O_2, thus demonstrating that the NO and H_2O_2 pathways may overlap to a greater extend than initially thought in HR induced cell death.

A protective molecule?

Finally, NO has been reported as both a cytotoxic and cytoprotecting molecule in plants (Beligni and Lamattina, 2001). This dual role may depend to a large extend on a tight spatio-temporal kinetic of cellular concentrations which will be governed by the production, displacement and removal of RNS (Noriega *et al*., 2007). NO was shown to function as an antioxidant and thus to protect plants from a variety of abiotic stresses such as drought, heat, salt or heavy metal stresses (Garcia-Mata and Lamattina, 2002; Uchida *et al*., 2002; Noriega *et al*., 2007; Tewari *et al*., 2008) and oxidative stress (Beligni and Lamattina, 2001; Dubovskaya *et al*., 2007). First, this protective effect may originate from direct detoxification of ROS by NO. Indeed, it is widely believed that NO can protect cells against oxidative stress by preventing the Fenton reaction by scavenging iron, thus avoiding the formation of hydroxyl radicals, one of the most phytotoxic oxygen radicals (Wink *et al*., 1995). Furthermore, the reaction between NO and $O_2\cdot^-$ which leads to $ONOO^-$ formation may help in reducing the adverse effects of oxygen radical accumulation. Second, the antioxidative properties of NO may rely on its ability to alter the plant antioxidant system. Indeed, it was recently demonstrated that NO could stimulate the expression of a heme oxygenase, which catalyses the conversion of heme to biliverdin IX with the concomitant release of CO and iron, and acts against oxidative stress in plants (Noriega *et al*., 2007). In addition, several studies have also reported an up-regulation of several antioxidant enzymes following treatment with low NO concentrations (Parani *et al*., 2004; Shi *et al*., 2005).

Finally, new insight into NO cytoprotective effects was recently provided by Belenghi *et al*. (2007). These authors reported that the *A. thaliana* metacaspase 9 (AtMC9) is constitutively *S*-nitrosylated *in vivo* at the catalytic Cys 147 residue. This posttranslational modification inhibits AtMC9 autoprocessing and proteolytic activity. This mechanism resembles those described for caspase 3 in mammalian cells. In resting cells, *S*-nitrosylation of the catalytic cysteine of caspase-3 maintains the enzyme in an inactive form (Mitchell *et al*., 2007). Upon apoptosis inducer action, thioredoxin mediates denitrosylation of

mitochondria-associated caspase-3, a process required for caspase-3 activation that promotes apoptosis (Benhar *et al*., 2008). Whether a similar thioredoxin-dependent de-*S*-nitrosylation contributes to AtMC9 up-regulation remains to be established.

Conclusion

NO has undoubtedly been an area on intense research over the past years. While the number of physiological processes involving NO is likely to grow, understanding of how this gas exerts its effects at the molecular level is still in its infancy. Clearly, there is no simple and uniform picture of the signaling function of NO (figure 1). Accumulating evidence now point out NO as one of the key messengers governing the control of Ca^{2+} homeostasis. The interaction between NO and Ca^{2+} operates in response to various stimuli in plants, suggesting that the cross-talk between both messengers is a basic transduction mechanism as reported in other organisms. Similarly, NO and ROS act in concert with protective or toxic effects as potential consequences, depending on the tight spatio-temporal kinetics of their respective production. It is however extremely difficult to predict the effects of the concerted action of NO and ROS, the main problems facing these studies being the current lack of drugs capable of selectively acting in one specie and our limited understanding of NO chemistry in plants. Finally, the ability of NO to modulate protein kinase activities represents another example of how NO mediates its action. The question of the physiological influence of NO/phosphorylation cascades remains, for the most, unanswered.

It is to be hoped that current and future studies will contribute towards the identification of *S*-nitrosylated, metal-nitrosylated and tyrosine nitrated proteins mediating NO signaling. Deeper insight into these NO-dependent post-translational protein modifications will not only permit the detailed characterization of the biochemical steps involved in NO control of the Ca^{2+}, ROS and protein kinases systems, but also will allow us to understand the physiological significance of the heterogeneous behaviours of NO in plants.

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